

AD-A178 309

IMMUNOLOGIC CONTROL OF DIARRHEAL DISEASE
DUE TO ENTEROTOXIGENIC ESCHERICHIA COLI
AND SHIGELLA

Annual Report

September 1986
(for the period January 1, 1985-December 31, 1985)

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Supported by:

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-83-C-3074

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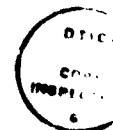
REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM	
1. REPORT NUMBER		2. GOVT ACCESSION NO.	
		AD-A178309	
3. TITLE (and Subtitle)		4. RECIPIENT'S CATALOG NUMBER	
Immunologic Control by Oral Vaccines of Diarrheal Disease Due to Enterotoxigenic <u>Escherichia coli</u> and <u>Shigella</u> .			
5. TYPE OF REPORT & PERIOD COVERED		6. PERFORMING ORG. REPORT NUMBER	
Annual Report Period 1/1/86-12/31/86			
7. AUTHOR(s)		8. CONTRACT OR GRANT NUMBER(s)	
Myron M. Levine, M.D., D.T.P.H.		DAMD 17-83-C-3074	
9. PERFORMING ORGANIZATION NAME AND ADDRESS		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS	
Center for Vaccine Development Command Department of Medicine University of Maryland, Baltimore, Maryland 21201		63750A.3M463750D808-AA-027	
11. CONTROLLING OFFICE NAME AND ADDRESS		12. REPORT DATE	
U.S. Army Medical Research and Development Command Fort Detrick, Frederick, MD 21701-5012		September, 1986	
13. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)		13. NUMBER OF PAGES	
		13	
14. SECURITY CLASS. (of this report)		15. DECLASSIFICATION/DOWNGRADING SCHEDULE	
Unclassified			
16. DISTRIBUTION STATEMENT (of this Report)			
Approved for public release; distribution unlimited.			
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)			
18. SUPPLEMENTARY NOTES			
19. KEY WORDS (Continue on reverse side if necessary and identify by block number)			
Enterotoxigenic <u>Escherichia coli</u> ; diarrheal disease; CFA/I, CFA/II; immunization; Salmonella; Shigella; Oral vaccine; Genetically engineered vaccine; enteropathogenic <u>Escherichia coli</u> .			
20. ABSTRACT (Continue on reverse side if necessary and identify by block number)			
<p>Travelers' diarrhea in several different clinical forms represents an important source of morbidity and loss of efficiency in United States military personnel who are deployed in less-developed areas of the world. The single most important etiologic agent of travelers' diarrhea is enterotoxigenic <u>Escherichia coli</u>, while the major cause of dysentery (loose stools with blood and mucus) is <u>Shigella</u>. Research has continued under this research contract to develop safe and effective immunizing agents to prevent these infections of military importance.</p>			

→ A candidate vaccine consisting of a non-enterotoxigenic E. coli that bears fimbrial colonization factors CS1 and CS3 was shown to stimulate markedly prominent SIgA anti-fimbrial antibodies in intestinal fluid after administration of a single oral dose. The geometric mean antibody level achieved after ingestion of a single oral dose of the live vaccine is approximately 10 times higher than is achieved with three 5 mg enteral doses of a purified CS1/CS3 fimbrial vaccine.

In order to provide broad protection, a vaccine against enterotoxigenic E. coli based on stimulating anti-colonization immunity must contain as antigens all the important colonization factors found in human enterotoxigenic E. coli strains. One common enterotoxigenic E. coli serotype that was not previously associated with known colonization factor fimbrial antigens is 0159:H4. A new fimbrial putative colonization factor has now been identified in 0159:H4 strains from throughout the world. The genes encoding these fimbriae are located on the same plasmid that encodes LT and ST enterotoxins. The fimbriae, which have been purified to homogeneity, are rigid filamentous organelles 6-7 nm in diameter.

In collaboration with investigators in the Department of Enteric Diseases at the Walter Reed Army Institute of Research, we have extensively tested the safety, immunogenicity and efficacy of a Shigella sonnei/Salmonella typhi bivalent attenuated vaccine (strain 5076-1C). Two lots of 5076-1C vaccine prepared at Forest Glen provided highly significant protection against challenge with pathogenic S. sonnei in studies in volunteers. A third lot failed to provide significant protection. Studies are underway to determine the factors that must be present to assure potency of this promising vaccine and to eliminate lot-to-lot variability in efficacy. If this can be accomplished, the safety level of protection conferred by strain 5076-1C are so impressively high (with effective lots of vaccine) that field trials of efficacy are indicated to assess the protection conferred by the vaccine under natural conditions of challenge.

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SUMMARY

Travelers' diarrhea in several different clinical forms represents an important source of morbidity and loss of efficiency in United States military personnel who are deployed in less-developed areas of the world. The single most important etiologic agent of travelers' diarrhea is enterotoxigenic Escherichia coli, while the major cause of dysentery (loose stools with blood and mucus) is Shigella. Research has continued under this research contract to develop safe and effective immunizing agents to prevent these infections of military importance.

A candidate vaccine consisting of a non-enterotoxigenic E. coli that bears fimbrial colonization factors CS1 and CS3 was shown to stimulate markedly prominent SIgA anti-fimbrial antibodies in intestinal fluid after administration of a single oral dose. The geometric mean antibody level achieved after ingestion of a single oral dose of the live vaccine is approximately 10 times higher than is achieved with three 5 mg enteral doses of a purified CS1/CS3 fimbrial vaccine.

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I. BACKGROUND

Diarrheal diseases represent one of the principal causes of morbidity and loss of efficiency among United States military personnel deployed in less-developed areas of the world (1-8). It is critical to develop measures to diminish the attack rate, severity of illness and loss of efficiency due to travelers' diarrhea in U.S. military personnel deployed in less-developed regions of the world of geopolitical importance.

The single most common etiologic agent causing travelers' diarrhea in U.S. adults who travel to less-developed areas is enterotoxigenic Escherichia coli (ETEC) (9-12). Shigella, the cause of bacillary dysentery, is often the second or third most frequently isolated agent of adult travelers' diarrhea (9). Although the incidence of diarrheal disease due to Shigella is appreciably lower than due to ETEC, the severity of the average case of clinical Shigella infection is notably greater; consequently, the soldier with Shigella illness is lost from duty for a longer period of time. Furthermore, Shigella infections can be transmitted from person to person by direct contact by means of very low infective inocula (as few as 10 Shigella organisms can cause clinical illness) (13). In contrast, ETEC infections are typically transmitted by contaminated food and water vehicles and require much larger inocula (circa 10^6) to cause illness (14-17). It is thus obvious that both ETEC and Shigella infections are of paramount military importance.

The Center for Vaccine Development has undertaken a long-term program to develop effective immunizing agents to prevent ETEC diarrhea. This program has involved studies of the pathogenesis of ETEC diarrhea and of the immune response to infection. It has also involved the development and evaluation of several vaccine candidates to prevent ETEC diarrhea (18-22). Several of the studies on the pathogenesis and prevention of

ETEC diarrhea represent collaborative projects with the Department of Gastroenterology of the Walter Reed Army Institute of Research.

Studies on ETEC during the past contract year have resulted in the identification and characterization of a new fimbrial colonization factor found in O159:H4 strains from throughout the world. Other studies have compared the intestinal SIgA anti-fimbrial antibody response following oral or enteral immunization with purified fimbriae (CS1 and CS3) or following oral immunization with a non-enterotoxigenic E. coli prototype vaccine strain bearing CS1 and CS3 fimbriae.

During the past contract year investigators at the Center for Vaccine Development have collaborated closely with Drs. S.B. Formal and T.L. Hale in the Department of Enteric Diseases of WRAIR on the development of a candidate vaccine to prevent Shigella sonnei disease. This vaccine strain, 5076-1C, was constructed at WRAIR by introducing the 120 Md plasmid from S. sonnei that encodes the production of sonnei O antigen into attenuated Salmonella typhi vaccine strain Ty21a. Strain 5076-1C has been extensively evaluated for safety, immunogenicity and efficacy in volunteer studies at the CVD.

Another area that has proven fruitful involves further studies of the pathogenicity of enteropathogenic E. coli of classical serotypes (EPEC) (23-28). The pathogens are characterized by their plasmid-encoded ability to adhere to HEp-2 cells with formation of clusters of adherent bacteria (so-called localized adherence). A DNA probe has been prepared from the plasmid that encodes localized adherence and has proven to be extremely useful in identifying EPEC and in studying their epidemiology. This represents a significant improvement over the previous main diagnostic tool, multi-step O serogrouping.

II. ENTEROTOXIGENIC ESCHERICHIA COLI

A. Studies with Candidate Vaccines against ETEC

A summary of our progress in evaluation of various oral vaccine candidates intended to stimulate ant-CS1 and ant-CS3 fimbrial intestinal antibody is contained in APPENDIX A. The most impressive results have been obtained with a prototype live oral vaccine strain, E1392/75-2A, of serotype O6:H16 that elaborates both CS1 and CS3 colonization factor fimbriae but does not possess the genes to produce LT or ST. A group of volunteers were vaccinated with a single oral dose of E1392/75-2A and were then challenged one month later with a wild ETEC strain of serotype O139:H28 that produces CS1 and CS3 and elaborates both LT and ST; a comparable group of control volunteers were also challenged. Results are shown in Table 1. While 6 of 6 controls developed diarrhea, only 3 of 12 vaccinees had diarrhea ($p < 0.005$) and the illness was milder. Bacteriologic studies (Table 2) showed that the ETEC challenge strain was recovered from the proximal small intestine in high titer from 5 of 6 controls but from only 1 of 12 vaccinees (who had only 10^1 organism/ml) ($p < 0.0004$). These data suggest that the mechanism of protection was by anti-fimbrial antibody preventing the pathogenic ETEC from colonizing in the proximal small intestine.

Table 3 shows the geometric mean SIgA antibody titers of the vaccinees before vaccination, after vaccination and immediately prior to challenge. In table 4 these titers are compared with the local SIgA anti-CS1/CS3 titers following enteral immunization with three 5 mg doses of purified CS1/CS3 vaccine.

B. A New Fimbrial Colonization Factor in ETEC of Serogroup O159

The work summarizing the discovery and characterization of a new fimbrial colonization factor in human ETEC strains of serotype O159:H4 is contained in APPENDIX B.

III. CLINICAL STUDIES WITH BIVALENT S. TYPHI/S. SONNEI VACCINE 5076-1C

A. Studies with Vaccine Prepared at the WRAIR Vaccine Production Facility at Forest Glen

Studies have been carried out with 5076-1C vaccine prepared, lyophilized and formulated at Forest Glen as well as with two lots of vaccine prepared, lyophilized and formulated at the Swiss Serum and Vaccine Institute in Berne, Switzerland. A summary of results obtained from clinical studies with the three lots of vaccine prepared at Forest Glen are contained in APPENDIX C. It is apparent that two lots of vaccine prepared at Forest Glen provided significant protection against shigellosis in experimental challenge studies. The reason for the lack of efficacy of the third lot of vaccine prepared at Forest Glen (intended for field trials in Israel, Chile and Thailand) is not yet clear. Preliminary evidence from studies being carried out at Forest Glen suggests that the lack of efficacy of the one lot may relate to certain properties of the carrier Ty21a strain. The apparent deficiency of the carrier strain in this lot seems to be related to the degree of flagellation of the bivalent attenuated strain.

B. Studies with Vaccine prepared at the Swiss Serum and Vaccine Institute

Two lots of vaccine prepared at the Swiss Serum and Vaccine Institute have also been tested in volunteer studies at the CVD. These lots of vaccine did not provide significant protection (Table 5). Preliminary studies being carried out at Forest Glen suggest that these two lots, like the one unprotective lot prepared at Forest Glen contain organisms that are deficient in flagellar H antigen by both electron microscopy and by serologic examination.

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TABLE 1

EFFICACY OF A SINGLE DOSE OF LIVE ORAL E. COLI VACCINE E1392-75-2A
(06:H16,CS1,CS3) IN PROTECTING AGAINST DIARRHEA FOLLOWING CHALLENGE WITH
 5×10^9 E. COLI F24377A (0139:H28, LT⁺/ST⁺, CS1,CS3)

<u>Group</u>	<u>Diarrheal Attack Rate</u>	<u>Severity of Diarrhea per Ill Volunteer:</u>	
		<u>Mean No. Stools</u>	<u>Mean Stool Volume</u>
Controls	6/6*	8.8 (2-18) ⁺	1147 ml (315-1855)
	p<.005		
Vaccinees	3/12	3.7 (2-5)	713 (220-1110)

*No. Ill/No. challenged.
⁺(Range)

TABLE 2

BACTERIOLOGIC FINDINGS IN K1389-75-2A VACCINEES AND
CONTROLS FOLLOWING CHALLENGE WITH ENTEROTOXIGENIC E. COLI
STRAIN E24377A (0139:H28, Lr⁺/St⁺, CS1,CS3).

<u>Group</u>	<u>Duodenal Cultures</u>	<u>Stool</u>
Controls	5/6 ^a (7x10 ³) ⁺	6/6 (8x10 ⁸)
Vaccinees	1/12 (10 ¹)	12/12 (1x10 ⁸)

p<.004

^aNo. positive/No. volunteers challenged.

⁺(Mean No. E. coli 24377A per gm stool or
Duodenal fluid).

TABLE 3

IMMUNOGENICITY OF LIVE ORAL NON-ENTEROTOXIGENIC E. COLI VACCINE

BEARING CFA/II FILTRIAE E1392/75-2A

<u>Antigen</u>	Significant Rises In Intestinal SIgA Antibody	Geometric Mean Titer		
		<u>Pre Vaccination</u>	<u>Post Vaccination</u>	<u>Pre- Challenge</u>
CFA/II (CS1, CS3)	10/10	5	416	315
OS	10/10	6	91	165
O139	0/10	2	2	2
LT	0/10	2	2	2

TABLE 4

COMPARISON OF THE INTESTINAL SIGA ANTI-FIMBRIAL ANTIBODY RESPONSE
 FOLLOWING ORAL IMMUNIZATION WITH A SINGLE DOSE OF LIVE ORAL VACCINE OR
 THREE ENTERAL DOSES OF PURIFIED CFA/II FIMBRIAE (CS1,CS3)

<u>Dose of Live Oral Vaccine Strain*</u>	<u>Seroconversion Rate for SigA Anti-CFA/II</u>	<u>Reciprocal Geometric Mean Titer</u>	
		<u>Pre</u>	<u>Post</u>
10 ¹⁰ organisms	10/10	5	416
<u>Purified CFA/II Fimbriae (CS1,CS3)</u>			
5 mg X 3	4/5	5	40

*E1392/75-2A (06:H16, LT⁻/ST⁻, CS1,CS3)

TABLE 8

EFFICACY OF ATTENUATED SALMONELLA TYPHI/SHIGELLA SONNEI BIVALENT VACCINE
STRAIN 5076-1C IN PROTECTING VOLUNTEERS AGAINST EXPERIMENTAL CHALLENGE
WITH PATHOGENIC SHIGELLA SONNEI*

<u>Type of illness</u>	<u>Attack rates (%)</u>		<u>P</u>
	<u>Controls</u> <u>(N=18)</u>	<u>Vaccinees</u> <u>(N=21)</u>	
Diarrhea	17	14	NS
Dysentery	22	10	NS
Fever:			
<u>>100°F</u>	22	19	NS
<u>>100°F</u>	17	19	NS

*Vaccine lyophilized at Swiss Serum and Vaccine
Institute after cultivation in broth.

CONTRACT-RELATED PUBLICATIONS FOR THIS CONTRACT YEAR

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against enteric infections. IXth International Congress of Infections
and Parasitic Diseases. Munich, July 20-26, 1986.

APPENDIX A

FIMBRIAE (PILI) ADHESINS AS VACCINES

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Certain fimbriae (pili) found only on enterotoxigenic Escherichia coli (ETEC), such as colonization factor antigens I and II (CFA/I, II) and E8775 fimbriae serve as virulence properties by allowing attachment of ETEC to enterocytes in the proximal small intestine (1). Veterinary studies have shown that antibody directed against analogous fimbriae in animal ETEC can suppress bacterial attachment and prevent diarrhea (2-5). We have studied ways to stimulate intestinal secretory IgA anti-CFA/II in animals and man using purified fimbriae or a live attenuated, non-enterotoxinogenic E. coli strain as oral vaccines (1,6,7). Eight 2.0 mg doses of purified CFA/II (CS1 and CS3 antigens) applied to the mucosa of an exteriorized loop of rabbit intestine stimulated a brisk SIgA anti-CFA/II response. Thirteen rabbits immunized orally with eight 0.8 mg doses of purified CFA/II fimbriae vaccine and 13 control rabbits were challenged with 10^{10} enterotoxigenic E. coli (RITARD method) bearing CFA/II. Diarrhea occurred in 8 of 13 of each group.

Ten volunteers were immunized with 2.0 mg of purified CFA/II orally twice weekly for four weeks (following suppression of gastric acid with cimetidine and NaHCO_3). Only 2 of 10 developed significant rises in intestinal SIgA or serum IgG anti-CFA/II. This cohort of volunteers was not significantly

protected, in comparison with unimmunized controls, when challenged with pathogenic ETEC; diarrhea occurred in 6 of 9 controls and 3 of 8 vaccinees with no difference in disease severity. In view of the excellent immunogenicity of this fimbriae vaccine in stimulating specific SIgA antibody when applied to the mucosa of exteriorized loops of rabbit intestine, we are distressed at the poor immune response following ingestion of multiple oral doses in man. We suspected that doxite cimetidine and NaHCO₃ treatment to neutralize gastric acid, perhaps the fimbrial protein was being adversely affected during its passage through the stomach. This seemed more probable when Schmidt et al (8) showed that gastric acid even at neutral pH, adversely affects fimbrial protein. Accordingly, we elected to administer three 5 mg doses of the CFA/II fimbrial vaccine directly into the duodenum via intestinal tube, thereby bypassing the stomach. Vaccine was given on days 0, 14, and 28 and intestinal fluid was collected to measure specific anti-fimbrial antibody on days 0, 14, 28 and 35. Given by this route, the CFA/II fimbrial vaccine stimulated significant rises in SIgA anti-CFA/II antibody in four of five vaccinees (Table 1).

Table 1. Significant rises in intestinal fluid SIgA antibody to CFA/II (CS1, CS3) antigens in volunteers given three 5 mg doses of CFA/II fimbrial vaccine via intestinal tube on days 0, 14 and 28:

Volunteer	SIgA Anti-CFA/II Titer ^a	
	Pre	Peak
1	4	4
3	<4	16
5	<4	1024
6	16	256
7	4	16

^ameasured by IgA-ELISA

These data emphasize the importance of protecting fimbrial vaccine proteins as they transit the human stomach. Research will have to be undertaken to identify practical and effective delivery systems.

Nineteen volunteers were fed a single 10^9 , 10^{10} or 6×10^{10} organism dose of a non-toxicogenic, CFA/II-positive O8:H16 strain (E1392-75-2A) as an oral vaccine (6,7). Two developed mild diarrhea, all had positive coprocultures and most had positive duodenal fluid cultures. Paired pre- and post-vaccination intestinal fluids were available from 14 of the 19 volunteers to measure local SIgA antibody. Significant rises in intestinal SIgA antibody to CFA/II antigens were detected in 11 of 14 including 6 of 6 who ingested a dose of 6×10^{10} vaccine organisms. Twelve volunteers who ingested a single 5×10^{10} organism dose of E1392-75-2A live oral vaccine and six unimmunized control volunteers were challenged with 5×10^8 enterotoxigenic E. coli of a distinct O:H serotype (O139:H28, LT+/ST+) but bearing the identical CFA/II antigens (CS1 and CS3) as the vaccine strain. Diarrhea occurred in 6 of 6 controls but in only 3 of 12 vaccinees ($p < .005$). Although the challenge strain was excreted in the same concentration in stool cultures of the two groups, a significant difference was noted in duodenal fluid cultures. The enterotoxigenic challenge strain was recovered from 5 of 6 controls (mean 7×10^3 E. coli/ml) but from only 1 of 12 vaccinees (10^1 E. coli/ml) ($p < .004$). These studies point to the superiority of live oral vaccines in stimulating anti-fimbrial antibody.

Acknowledgements: These studies were supported by research contracts NO1AI42553 from the National Institute of Allergy and Infectious Diseases and DAMD 17-78-C-8011 from the U.S. Army Medical Research and Development Command.

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APPENDIX B

A NEW FIMBRIAL PUTATIVE COLONIZATION FACTOR OF ENTEROTOXIGENIC

ESCHERICHIA COLI SEROTYPE O159:H4: PURIFICATION,

MORPHOLOGY, AND GENETICS

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This work was supported by grant NO1-AI-12886 from the National Institute
of Allergy and Infectious Disease and U.S. Army contract DAMD 17-83-C3074.

ABSTRACT

The ability to colonize the small intestine is essential for the pathogenesis of diarrhea due to enterotoxigenic Escherichia coli (ETEC). Colonization is mediated by fimbriae (pili) of which there are several antigenically distinct types, including CFA I, CFA II (CS1, CS2, and CS3) and PCP8775 (CS4, CS5, and CS6). These fimbriae are associated with certain ETEC O serogroups. Serogroup O159 has lacked a known colonization factor. We have found a distinct plasmid-encoded fimbria composed of 19 Kd protein subunits associated with ETEC serotype O159:H4. Rabbit antibody against this purified fimbria reacted with a single 19 Kd protein band on Western immunoblot of sheared cell preparations. The rabbit antibody, treated with colloidal gold-labelled goat anti-rabbit IgG, bound specifically to fimbriae when cells were examined in the electron microscope. Six of 10 available strains of ETEC O159:H4 from Europe, Bangladesh, and Africa expressed this fimbria; its true prevalence among ETEC is unknown. This putative colonization factor of O159:H4 joins other ETEC fimbriae as potentially useful immunogens against human diarrhea.

INTRODUCTION

Enterotoxigenic Escherichia coli (ETEC) is a leading cause of diarrhea and dehydration in children less than two years of age in developing countries (1,2); ETEC is the most common cause of travelers' diarrhea (3,4) and is occasionally responsible for outbreaks of food- and water-borne disease (5,6). Investigations of the pathogenesis of ETEC diarrhea have identified factors essential for virulence which might be manipulated to produce a vaccine. These virulence factors include fimbrial colonization factors, which allow the organism to adhere to epithelium of the proximal small intestine, and heat-stable (ST) and/or heat labile (LT) enterotoxins. The ability of these toxin-producing E. coli to adhere to intestinal epithelium and resist the clearing action of peristalsis is essential for virulence. In volunteer studies, diarrhea did not occur after ingestion of an organism that produced LT but had lost a plasmid encoding a fimbrial colonization factor and ST; the plasmid-containing fimbriate parent strain did cause diarrhea (7).

The ability of some ETEC strains to cause mannose-resistant hemagglutination led to the identification of several colonization factors of human ETEC. These include CFA I (colonization factor antigen I), CFA II complex (which consists of 3 antigenically distinct fimbriae designated CS1, CS2, and CS3), PCF8775 (which also has 3 distinct fimbriae designated CS4, CS5, and CS6), and perhaps a fimbria of strain 280-1 (8-13). These colonization factor antigens are associated with certain O serogroups: O25, O63, O78, O128 (CFA I), O8, O6, O60, O65 (CFA II), and O25, O27, O115, O148, and O167 (PCF8775). All these colonization factors are

fimbriae, the genes for which are plasmid-encoded. The genes encoding ST (and sometimes LT) are found on the same plasmid with CFA I or CS1, CS2, and CS3. The same is usually, but not invariably, true of genes for CS4, CS5, and CS6.

When large collections of ETEC have been screened for the presence of the known colonization factors, their prevalence has varied widely (11,14-18). When strains isolated from patients with diarrhea, but shown to lack CFA I or CFA II, were fed to volunteers, the volunteers nevertheless became ill with diarrhea (19). These observations prompted the suggestion that other, antigenically distinct, colonization factors must be present on these strains and on other ETEC lacking known colonization factors. Indeed, ETEC of serogroup O159 are commonly associated with diarrhea in many geographic areas but do not cause mannose-resistant hemagglutination and lack a known colonization factor. In this report we describe an antigenically distinct fimbrial colonization factor associated with the common ETEC serotype O159:H4.

MATERIALS AND METHODS

Bacterial strain. Enterotoxigenic E. coli strain 350C1 serotype O159:H4 was chosen for study (14). This serotype has been commonly associated with diarrhea and strain 350C1 does not agglutinate with antisera against CFA I, CFA II, or PCF8775. It is a non-hemagglutinating strain which lacks type 1 pili when grown on solid agar. The strain produces both ST and LT (14).

Purification of fimbriae. A suspension of 350C1 was streaked onto aluminum pans (32x42x2 cm) containing 1 liter of CFA agar (1% casein acids, 0.15% yeast extract, 0.005% MgSO_4 , and 0.0005% MnCl_2 , 2% agar, pH 7.4) (20) and incubated overnight at 37°C. The bacterial cells were harvested, suspended in 20 ml phosphate buffered saline pH 7.2 (PBS), and sheared for 90 seconds on ice in a Sorvall Omnimixer at setting 5. Cells and particulate debris were pelleted by centrifugation at 8,000xg for 30 minutes; outer membrane vesicles were removed by centrifugation at 39,000xg for 30 minutes. The supernatant was collected and centrifuged at 180,000xg for 1.5 hr in a Beckman L8-80 centrifuge. The pellet, containing fimbriae, was suspended in 0.1 M Tris, 0.2 mM EDTA with a magnetic stirrer at 4°C.

This suspension was applied to a self-generating isopycnic cesium chloride gradient with 1.5% w/v sodium N-lauroyl sarcosine of density 1.3 g/cm^3 by ultracentrifugation at 117,000xg for 44 hr. Opaque bands visible under strong illumination were collected by tube puncture and dialyzed overnight in PBS at 4°C. The presence of fimbriae was monitored by electron microscopic examination and examination of fimbrial subunits by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10).

SDS-PAGE. SDS-PAGE gels were prepared by the method of Laemmli (21) with modifications of Lugtenberg (22) and Shapiro (23). Stacking and separating acrylamide gels of 5%, pH 6.9 and 12.5%, pH 8.7 respectively were prepared in a 93 x 102 x 1.5 mm water-cooled mini-gel apparatus (Hoefer Scientific). Electrophoresis buffer was the tris-glycine preparation of Ornstein (24) and Davis (25) with 0.1% SDS. Samples were boiled for 5 minutes with equal volumes of 62.5 mM Tris base, 2% SDS, 10%

glycerol, 0.05% bromphenol blue, and 5% 2-mercaptoethanol and approximately 1 ug per lane loaded by syringe into wells. End wells contained prestained molecular weight markers (Bethesda Research Laboratory) for estimation of size and electrophoresis time. Electrophoresis was conducted at 25 vmp until samples stacked, then 35 vmp until markers were well resolved. Gels were stained with Coomassie brilliant blue R-250.

Anti-fimbria antiserum. An adult male rabbit weighing 2 kg was inoculated subcutaneously between the scapulae and into the rear footpads with purified fimbriae in Freund's complete adjuvant. At 8 weeks, a booster injection of fimbriae with Freund's incomplete adjuvant was given subcutaneously. The rabbit was bled 2 weeks later. Specific antisera was prepared by multiple absorptions with nonfimbriate cells grown at 18°C (8).

Bacterial agglutination. Slide agglutination of bacterial cells was performed by mixing a drop (0.05 ml) of antiserum with a suspension of colonies on a toothpick at room temperature.

Immunoblotting. Crude shear preparations and purified fimbriae were electrophoresed in duplicate on SDS-PAGE gels, one of which was stained with Coomassie blue and the other electoblotted onto nitrocellulose. Western blots were developed as described by Towbin et al (26) with the modifications of Battsiger et al (27). Absorbed rabbit antiserum was diluted in 0.5% Tween-20 in PBS and incubated with the nitrocellulose filter overnight at 4°C. Filters were washed 3 times for 10 minutes each in diluent and agitated in 1:1000 goat anti-rabbit IgG conjugated to horse radish peroxidase (Cooper Biomedical, Malvern, Pa.) for 2 hours at room temperature. Filters were again thoroughly washed and developed with

hydrogen peroxide substrate and 4-chloro-naphthol chromagen. Dot immunoblots of whole cell preparations were performed by suspending cells from an overnight culture in PBS and dotting the suspension on nitrocellulose filters. These were incubated with absorbed rabbit antiserum, washed, reacted with goat anti-rabbit IgG, and developed as for the Western blot.

Electron microscopy. Bacterial cells were grown at 37°C on CFA agar, harvested, and examined directly with a Siemens Elmiskop IA transmission electron microscope operated at 80 Kv with negative staining by 2% phosphotungstic acid (10).

Immunolabelling with colloidal gold-labelled goat anti-rabbit antisera was performed (10). A drop of a washed suspension of strain 350C1 was placed on carbon-coated grids and allowed to partially dry under a light bulb for 5 minutes. Excess liquid was removed and the grid was placed face down on a drop of antiserum for 15 minutes. After washing in 3 successive drops of distilled water, the grids were placed on a drop of 10 nm-gold-labelled goat anti-rabbit serum (Janssen Pharmaceutical, Piscataway, N.J.) for 15 minutes (28). The grid was washed in 3 drops of distilled water. The grid was then stained with 2% phosphotungstic acid. Grids incubated without the primary antibody or with anti-CFA I antibody were used as controls.

Characterization of plasmid DNA. Plasmid DNA was extracted by the Birnboim alkaline extraction technique (29) and examined by electrophoresis on a 0.4% agarose gel. Plasmids were cured by multiple passages on tryptic soy agar (TSA) at 37°C and by incompatibility matings. Matings were performed by mixing suspensions of each parent and incubating the mixture overnight on a nucleopore filter on an L agar plate

(30). Transconjugates were recovered from the filter by plating on appropriate selective media.

The 27 Kdal plasmid was marked with drug resistance using ampicillin resistance transposon Tn801 and transformed into HB101. Cells were plated on L agar containing 200 ug/ml of ampicillin to select for ampicillin resistant transformants. Transformants were examined for fimbriae in the electron microscope.

DNA hybridization. Colony blots were performed under stringent conditions as described by Moseley (30). The LT-B DNA probe was prepared from pWD615 and the ST DNA probe from pSLM004 by nick translation with α -³²P dATP (New England Nuclear, Boston) (30).

ELISA for ST and LT. The direct culture method GM1 ganglioside enzyme-linked immunosorbent assay (ELISA) for LT (31) and monoclonal antibody ELISA for ST (32) were performed with ETEC 350C1 and its plasmid-cured derivatives.

RESULTS

Purification of fimbriae. Colonization factor antigens are characteristically expressed when organisms are grown at 37°C and not when grown at 18°C (19). Crude shear preparations of O159:H4 cells grown at 37°C on CFA agar were subjected to SDS-PAGE and compared to shear preparations of O159:H4 cells grown at 18°C. The protein banding patterns were identical except for a 19 Kd band present in the 37°C cell preparation (Figure 1, lane B) that was absent from the 18°C cell preparation (Figure 1, lane A). Differential centrifugation and isopycnic CsCl gradient banding yielded purified fimbriae. On SDS-PAGE this preparation produced a single polypeptide subunit band of 19 Kd (Figure 1, lane C).

Electron microscopy. Examination of strain 350C1 grown at 37°C on CFA agar revealed rigid filamentous fimbriae of 6-8 nm diameter (Figure 2). Cells grown at 18°C on CFA agar lacked fimbriae. Figure 3 shows purified fimbriae which are morphologically identical to those seen on the cell surface.

Figure 4 shows strain 350C1 treated with absorbed rabbit serum against fimbriae and reacted with gold-labelled goat anti-rabbit IgG. Only fimbriae, not flagella or background, are labelled with gold, indicating the specificity of the absorbed rabbit serum for this bacterial organelle.

Immunologic studies. Neither pre-immune, immune, or immune absorbed rabbit serum agglutinated whole 350C1 cells grown on CFA at 37°C.

Figure 5 is an immunoblot of the crude and purified fimbrial preparations shown in Figure 1. Pre-immune serum did not react with any of the proteins in these preparations. Absorbed immune rabbit serum reacted with a single band of 19 Kd in the shear preparation of strain 350C1 (Figure 5, lane B) and with purified fimbriae of strain 350C1 (Figure 5, lane C). This specific antiserum against 350C1 fimbriae did not react with the subunits of CS1, CS3, CFAI (Figure 5, lanes E-G), or PCF8775 (data not shown).

Plasmid studies. The plasmid content of strain 350C1 included 2 plasmids of nearly identical size, 27 and 28 Mdal (Figure 6, lane B). The derivative cured of the 28 Mdal plasmid (Figure 6, lane C) produced both ST and LT and expressed fimbriae on electron microscopic examination. The derivative cured of the 27 Mdal plasmid (Figure 6, lane A), however, was non-toxicogenic by colony blot with ST and LT gene probes and by ELISA for ST and LT and lacked fimbriae, indicating that the genes for ST and LT and for the fimbria are coded on the 27 Mdal plasmid. SDS-PAGE of a shear preparation of this derivative lacked the 19 Kd fimbrial subunit (Figure

1, lane D). HB101 transformed with the 27 Mdal plasmid marked with Tn801 expressed fimbriae that were visualized in the electron microscope.

Prevalence of 350C1 fimbriae among ETEC. ETEC in the Center for Vaccine Development collection were screened by dot immunoblot of whole cells and Western blot of sheared cell preparations. Six of 10 available ETEC O159:H4 from Europe, Bangladesh, and Kenya (including several strains kindly provided by Frits Orskov, WHO Escherichia Collaborating Centre, Statens Serum Institut, Copenhagen, Denmark) reacted with specific absorbed antiserum against the fimbriae of strain 350C1 by both dot blot of whole cells and Western blot of shear preparations. None of 8 ETEC O159 of other H serogroups and none of 3 ETEC O27 reacted with this antiserum.

DISCUSSION

Studies of the human response to ETEC have shown that infection provides protection against future challenge with the homologous strain (33). Antibodies to any of several products or structural components of E. coli might mediate this protection. Infection-derived antibodies to LT alone were not able to provide protection in volunteer studies (33) and antibodies to ST are not produced after natural ETEC infection. The mechanism of protection does not involve bactericidal effects, since stool cultures are positive for ETEC in protected individuals after an oral challenge (33).

Local secretory IgA to colonization factor antigens may be the key to protection. Purified K88, K99, and 987P fimbriae from animal ETEC have been used as successful vaccines against colibacillosis in farm animals

(34,35). Studies of the human response to colonization factor antigens have shown that when volunteers are fed organisms with colonization factors, significant rises in secretory IgA antibody and serum antibody to the colonization factor are stimulated, indicating their expression in vivo and immune recognition by the host (7,10,36). Volunteers given a single dose of ST⁻ LT⁻ CS1⁺ CS3⁺ ETEC O6:H16 as a live oral vaccine developed intestinal secretory IgA against CS1 and CS3 and were significantly protected when challenged with an ST⁺ LT⁺ CS1⁺ CS3⁺ strain of serotype O139:H28 (36), suggesting that antibody to the only common antigens, CS1 and CS3 fimbriae, was adequate for protection. Stool cultures were positive in vaccinees and controls. However, jejunal fluid cultures were positive in only 1 of 12 vaccinees but in 5 of 6 controls ($p < 0.004$), indicating that the site of protection was the jejunal mucosa where colonization was prevented (36,37).

Certain O serogroups have been repeatedly associated with ETEC diarrhea in humans throughout the world; these include O6, O8, O25, O27, O63, O78, O80, O85, O115, O128, O148, O159, and O167. These are the serogroups representing the majority of ETEC isolated from most severe (e.g., hospitalized) cases of diarrhea. Conspicuously, serogroups O27, O115, O148, O159, and O167 were not associated with CFA I or II. The recently described PCF8875 complex of CS4, CS5, and CS6 fimbrial antigens has been found to occur in O27, O115, O148, and O167 serogroups. O159 has been the last common and important serogroup without a colonization factor. A distinct fimbrial colonization factor has now been identified in this "holdout." The way is clear, in theory, to prepare a fimbrial antigen vaccine of truly broad spectrum that will prevent illness due to all the O serogroups associated with more severe forms of ETEC diarrhea.

Preliminary studies in volunteers suggest that orally administered purified fimbriae may undergo proteolysis in the stomach or intestine or be less potent immunogens than when fimbriae are attached to living organisms (37). A live, oral vaccine strain (or strains) engineered to express several colonization factor antigens might provide protection against a broad spectrum of ETEC; an attenuated S. typhi strain such as Ty21a might be a useful host strain (38).

We propose the designation PCF (putative colonization factor) O159:H4 for this new fimbria. The definitive role of PCF O159:H4 in the pathogenesis of diarrhea in man needs to be evaluated, as does the role of CFA II fimbriae and PCF8775 fimbriae. In the native O159:H4 strain, genes for the toxins and fimbriae are carried on the same plasmid so that simple curing of the plasmid does not allow separate evaluation of these virulence factors.

The available data, albeit preliminary, incriminating PCF O159:H4 as a colonization factor are the same as those that exist for CFA II and PCF8775. Volunteer studies with fimbria-minus variants of strains with these colonization factors have not been carried out. Furthermore, in the volunteer studies with CFA I, the plasmid-minus variant that failed to cause diarrhea in volunteers had lost the ability to produce ST as well as CFA I when the plasmid was cured. Thus for none of the human putative colonization factors has a definitive study been done to conclusively demonstrate their pathogenic role. Future studies to inactivate the fimbrial genes by genetic engineering will allow precise evaluation of the importance of these fimbriae, independent of toxin, in animal and human models of disease.

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Figure 1. SDS-PAGE of crude and purified fimbria preparations. Lane A contains proteins of a shear preparation of E. coli 0159:H4 strain 350C1 grown on CFA agar at 18°C, conditions which inhibit expression of fimbriae. Lane B contains proteins of a shear preparation of the same organism grown at 37°C, conditions which encourage expression of fimbriae; an additional band of 19 Kd representing fimbrial subunits is seen (arrow). Lane C shows purified 0159:H4 fimbrial subunits. Lane D shows proteins of a shear preparation of the 0159:H4 fimbrial subunits. Lane D shows proteins of a shear preparation of the 0159:H4 strain cured of the plasmid that encodes fimbriae; no 19 Kd band is visible. Lanes E, F, and G show purified subunits of other ETEC fimbriae CS1, CS3, CFA I, respectively, for comparison.

Figure 2. ETEC 0159:H4 strain 350C1, negatively stained with 2% phosphotungstic acid. Filamentous fimbriae of 6-7 nm size are visible. 55,900 X.

Figure 3. Purified ETEC 0159:H4 fimbriae, negatively stained with 2% phosphotungstic acid. 78,000 X.

Figure 4. ETEC 0159:H4 labelled by the immunogold technique. Cells were incubated with rabbit anti-fimbriae serum, followed by 10 nm colloidal gold-labelled goat anti-rabbit serum. Cells were negatively stained with 2% phosphotungstic acid. Fimbriae, not flagella or other structures, are clearly labelled with gold particles. 49,400 X.

Figure 5. Western immunoblot of crude and purified ETEC 0159:H4 fimbriae. Lanes correspond to those in SDS-PAGE in Figure 1. Only lanes B and C containing crude and purified fimbrial subunits react with mono-specific anti-fimbriae serum.

Figure 6. Agarose gel electrophoresis of plasmid DNA of ETEC 0159:H4 strain 350C1. Lane B shows the native strain with 2 plasmids of 27 Mdal and 28 Mdal size. Lane C contains the plasmid of the derivative cured of the 28 Mdal plasmid; this strain produced ST, LT, and fimbriae. Lane A contains the plasmids of the derivative cured of the 27 Mdal plasmid; it did not produce ST, LT, or fimbriae. The new large molecular weight plasmid in lane A was introduced during incompatibility mating.



Figure 1.

A B C D E F G

19 ➤

Figure 2.



Figure 3.



Figure 4.

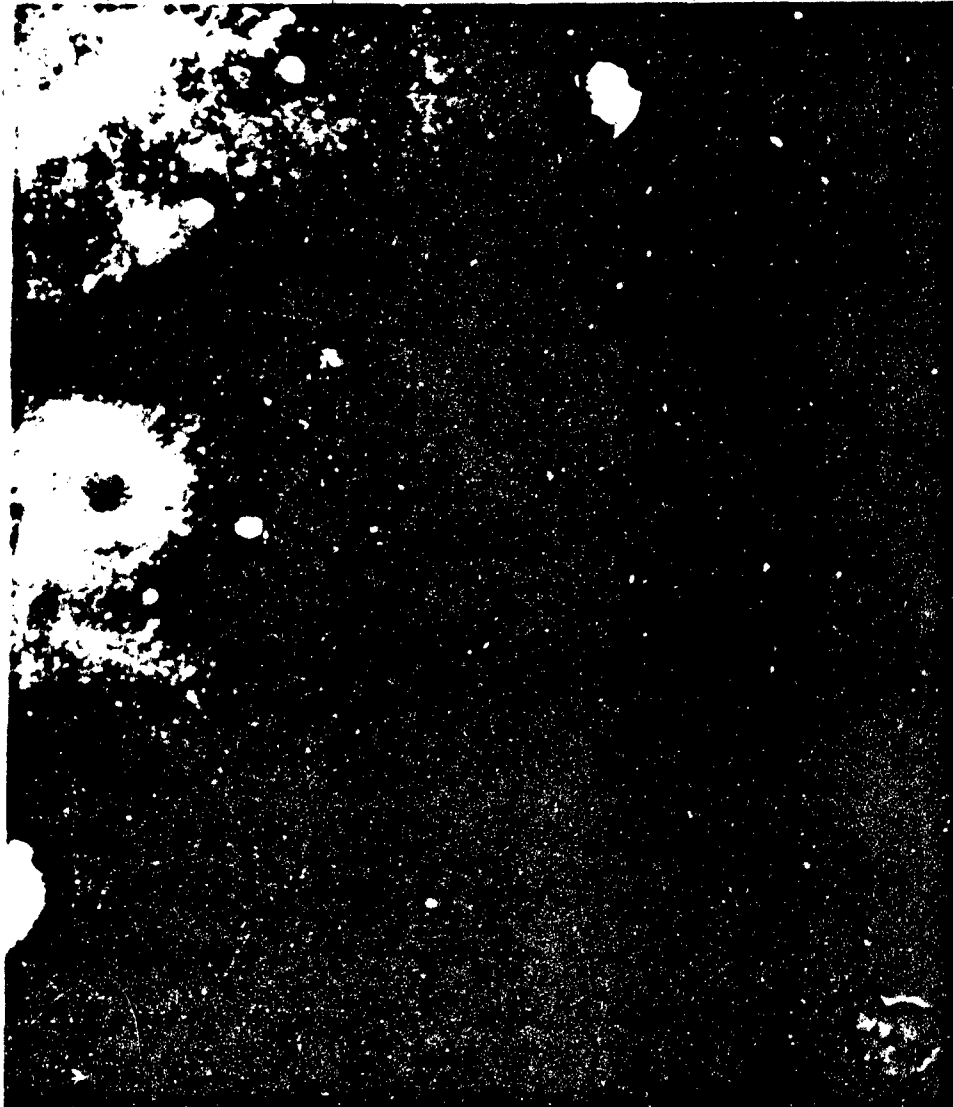
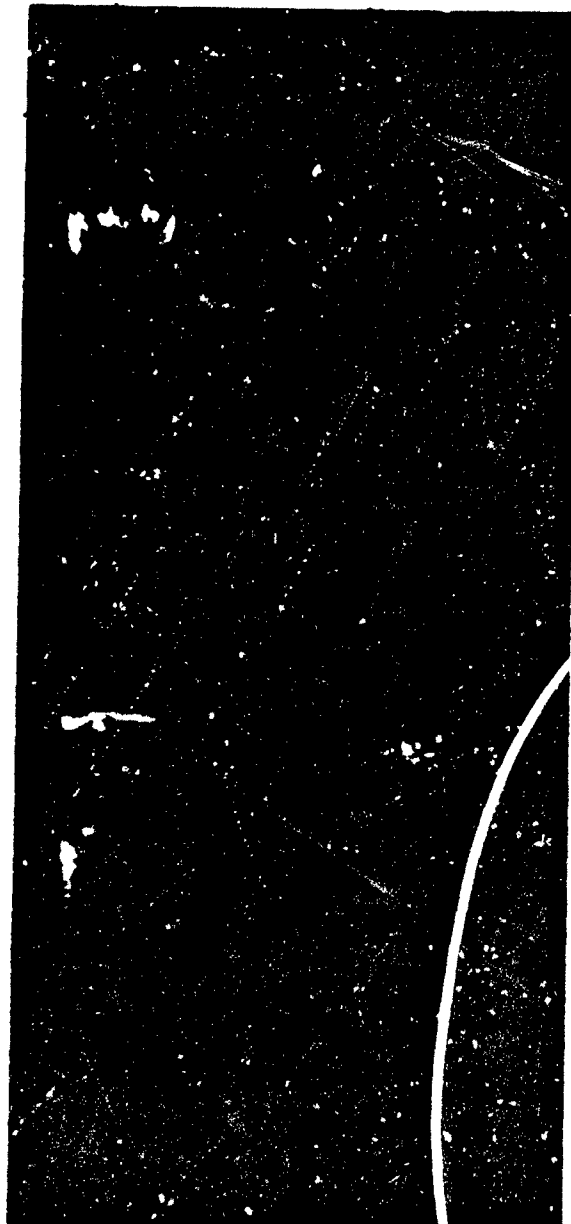


Figure 5.

A B C

28 Mdal ➤

Chr ➤



◀ **27 Mdal**

Figure 6.

APPENDIX C

Prevention of Shigellosis
by a Salmonella typhi - Shigella sonnei Bivalent Vaccine

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This study was approved by the human volunteer research committees at the University of Maryland Hospital and the National Institute of Allergy and Infectious Diseases. It was supported by contracts from the U.S. Army Medical Research and Development Command (DAMD 17-83-C-3074) and the National Institute of Allergy and Infectious Diseases (N01AI12666).

ABSTRACT

Attenuated Salmonella typhi strain Ty21a has been genetically modified to express the form I O polysaccharide antigen of Shigella sonnei, creating a possible bivalent live oral vaccine strain. Three doses of this strain (10^9 organisms per dose) were given to young adults who, along with unvaccinated controls, were challenged one month later with pathogenic S. sonnei. The vaccinated individuals had 40% protection against diarrhea and 56% efficacy against dysentery due to S. sonnei. Two of 3 vaccine lots evaluated provided higher levels of protection (53% against diarrhea and 71% against dysentery) but the third lot, which was prepared for a large scale vaccine field trial, demonstrated no protective efficacy. Vaccinated individuals manifested serum and local intestinal immune responses to S. sonnei lipopolysaccharide and the presence of specific serum IgA or IgG antibody prior to experimental challenge with pathogenic S. sonnei was apparently correlated with protection from illness. These results indicate that some lots of this bivalent vaccine strain provide significant protection against S. sonnei disease; however, the problem of lot to lot variability in efficacy must be overcome before practical use can be made of this vaccine.

Shigellosis, an important diarrheal illness worldwide, may be amenable to control by an effective vaccine that can be delivered to young children. Although parenteral vaccines comprised of killed Shigella organisms are not protective, some attenuated Shigella strains given orally have shown serotype-specific protection in experimental human challenge studies and in field trials (1-3). However, these attenuated Shigella vaccines have not reached widespread public health use because of occasional genetic instability (4), mild adverse reactions (5,6), and insufficient immunogenicity under some circumstances (7).

Considerable epidemiologic evidence exists that Shigella O antigens play an important role in stimulating protective immunity (1). The group and type-specific O surface antigens are encoded by genes which, when transferred into a recipient organism, can result in expression of these antigens along with those of the recipient strain. Formal et al (8) created a prototype bivalent vaccine strain by conjugal transfer of the 120-mdal S. sonnei plasmid, which encodes the form I O polysaccharide cell surface antigen, into the galE oral typhoid fever vaccine strain Salmonella typhi Ty21a. This recipient strain may be particularly suitable since it is safe and protective against typhoid fever in endemic areas (9-11).

The S. typhi-S. sonnei bivalent vaccine strain (5076-1C) has been shown to express both S. typhi and S. sonnei O antigens (8). Strain 5076-1C, which maintains the attenuation characteristics

(sensitivity to galactose) of Ty21a, was demonstrated to be safe and immunogenic on initial testing in humans (12). Furthermore, the strain stimulates an intestinal IgA response to both S. typhi Ty21a and S. sonnei form I antigens in rabbits (13). This report describes the clinical and immune response after vaccination of humans with strain 5076-1C and the protective efficacy of the vaccine against challenge with pathogenic S. sonnei.

MATERIALS AND METHODS

Vaccine. A streptomycin-resistant mutant of S. typhi Ty21a was the recipient for conjugal transfer of the form I S. sonnei plasmid as previously described (8). The resultant transconjugant strain, S. typhi 5076-1C, was harvested after growth on solid agar, dispensed into vials, and lyophilized at the Department of Biologic Research, Walter Reed Army Institute of Research. On the days of vaccination, vials of lyophilized vaccine (stored at 4C) were reconstituted with sterile distilled water to yield a concentration of $1-3 \times 10^9$ viable organisms. Three different lots of vaccine were used in volunteer studies. Lot 2 was used for the first two studies, lot 5 for the third study and lot 8, which was produced for a large scale field trial of vaccine efficacy, was used for the fourth study.

Volunteers. Participants were healthy young adults from the Baltimore community to whom all aspects of the study were

described in detail. The methods of medical screening, informed consent and medical care have been published (14-17). Vaccination was performed on an outpatient basis, while challenge studies were carried out in the isolation ward maintained by the Center for Vaccine Development in the University of Maryland Hospital. Three separate studies, involving approximately 20 volunteers each, were performed and the results pooled for analysis, since the methods utilized were identical.

Administration of Vaccine. The vaccine was administered orally with 2.0 g of NaHCO_3 mixed in 150 ml of water. Volunteers fasted for 90 minutes before and after ingesting the vaccine. Three doses of the strain were given to each vaccinee with 3-4 days separating each dose. The volunteers returned as outpatients 24 and 48 hours after vaccination and were questioned about possible reactions (e.g., fever, cramps, diarrhea).

To determine if the vaccine strain could be recovered, stool samples were collected 24 and 48 hours following each vaccine dose. They were heavily streaked on MacConkey agar with streptomycin (500 mcg per ml) and incubated for 24 h at 37C. Colonies were identified by agglutination with S. sonnei and Salmonella group D antisera.

Challenge Study. One month after receiving the third dose of vaccine, the group of vaccinees and a group of unvaccinated controls were challenged with approximately 500 pathogenic S. sonnei (70-80% were smooth form I colonies). The volunteers

ingested the organisms suspended in 45 ml of skimmed milk (reconstituted from powdered milk with distilled water). Volunteers fasted for 90 minutes before and after ingesting the challenge inoculum.

Volunteers were examined and interviewed daily for 120 hours before receiving a 10 day course of 2 tablets of trimethoprim (80 mg) and sulfamethoxazole (400 mg) every 12 hours. Volunteers who met the definition of diarrhea received antibiotics 24 hours after the onset of diarrhea.

All stools were collected in a sterilizable plastic pan that fit on the commode. Each stool was examined by a staff member and graded on a five point scale: grade 1, formed; grade 2, soft; grade 3, thick liquid; grade 4, opaque water; grade 5, clear watery (17). All stools were weighed and tested for blood by Hematest (Ames). Diarrhea was defined as the passage of 2 or more liquid (grades 3-5) stools within 48 hours and at least 200 g in weight, or a single liquid stool of 300 g or greater. Dysentery was defined as at least one liquid stool with blood. Temperatures were taken every 6 hours and repeated again in five minutes with a different thermometer if ≥ 100 F. Illness was considered the presence of diarrhea, dysentery, or fever (temperature ≥ 100 F).

Immunologic Studies. Vaccinees had sera obtained before and 9, 15, 21 and 28 days after vaccination. Vaccinees in the first and second studies also had jejunal fluid collected before and 15 days after the first dose of vaccine. Vaccinees and

controls participating in the challenge studies had sera collected before, and 8, 21, and 28 days after challenge with S. sonnei. Volunteers in the first three studies had intestinal fluid collected before and 7-8 days after challenge.

For collection of intestinal fluids, volunteers swallowed polyvinylchloride intestinal tubes, which were localized in the jejunum by distance (130 cm) and the appearance of bile-stained fluid of pH 6.0 or above. Jejunal fluid (70 ml) from each volunteer was centrifuged (8,000xg) to remove particulate material. SIgA was measured by radial immunodiffusion (18) as previously described (15). Aliquots of jejunal fluid were dispensed into polystyrene tubes and lyophilized. The fluids were reconstituted to a concentration of 20 mg of SIgA per 100 ml before testing for specific antibody.

Serum IgG and IgA and jejunal fluid SIgA antibody to S. sonnei O antigen were measured by enzyme-linked immunosorbent assay (ELISA). Purified S. sonnei O antigen (25ug/ml), 100 ul) was adsorbed to wells of polystyrene microtitration plates for 2 hrs. at 37C, followed by overnight at 4C. The wells were washed with phosphate-buffered saline (PBS) pH 7.2 and the remaining unbound plastic sites were blocked with 5% heat-inactivated fetal bovine serum in PBS for 1 hr at 37C. Wells were washed with PBS containing 0.05% Tween 20 (washing buffer). Serial two-fold dilutions of serum (beginning at 1:40) or jejunal fluid (beginning at 1:4), in washing buffer containing 1% heat-inactivated fetal bovine serum, were incubated overnight at 4C. After washing, alkaline phosphatase-conjugated goat anti-human IgG or

IgA was incubated in the wells for 1 hr at 37C and the ELISA completed sequentially with p-nitrophenyl phosphate and 3M NaOH (19). Optical density was read at 405 nm using a Titertek Multiskan-MC (Flow Laboratories, McLean, Va.). Four-fold increases in titer were considered significant.

Analysis: Statistical comparisons were done by chi square, Fisher's exact test (one tail) and Student's t test.

RESULTS

The bivalent strain was administered to 60 individuals without adverse reactions. Ten (17%) of the volunteers excreted strain 5076-1C in the two days after the first vaccine dose, as did 10 (18%) of 57 after the second dose and 5 (9%) of 57 after the third dose. Forty of these vaccinees, along with 38 unvaccinated controls, participated in subsequent challenge studies to determine vaccine efficacy.

In the challenge studies, 20 (53%) of the controls developed diarrhea and 19 (50%) dysentery, compared with 13 (32%) and 9 (22%) of the vaccinees, respectively (Table 1). This suggests a vaccine efficacy of 40% against diarrhea ($p = 0.06$) and 56% against dysentery ($p = 0.01$). The vaccinees also had 57% protection against febrile ($\geq 100^{\circ}\text{F}$) illness ($p = 0.006$), and 76% protection against more severe febrile illness ($\geq 101^{\circ}\text{F}$) ($p = 0.001$), which occurred in nearly half of controls. Those

vaccinees who developed diarrhea had similar mean numbers of diarrheal stools and total volume as ill controls. Stool cultures were positive after challenge in a similar high proportion of vaccinees and controls.

The first 3 studies were done with two different vaccine lots (numbers 2 and 5) and appeared to have excellent efficacy (71% protection against dysentery and complete protection against more severe febrile disease (Table 2). After these encouraging results, a third vaccine lot (Number 8) was prepared for a field efficacy trial and evaluated in volunteers. This vaccine lot did not appear to stimulate protective immunity (Table 2).

After receiving three doses of the bivalent strain, some vaccinees had serum IgG, or serum or local intestinal IgA responses to S. sonnei O antigen (Table 3). Of the three antibody responses, rises in local IgA titer were the most frequent. Unvaccinated controls after challenge with pathogenic S. sonnei had higher frequencies of serum IgG ($p = 0.003$) and IgA ($p < 0.001$) responses, but a similar frequency of local IgA response, than individuals following vaccination. Furthermore, the controls after challenge with pathogenic S. sonnei had higher frequencies of serum and local immune responses than did the vaccinees who participated in the experimental challenge studies (Table 3).

The vaccinees' immune responses to vaccination and the vaccinees' and controls' antibody status before experimental challenge were examined in order to identify immunologic correlates of clinical protection. The vaccinees who had a serum

IgA response to the bivalent strain tended to have a lower attack rate in the challenge study, but the differences were not significant (Table 4). The presence prior to challenge of serum IgA or IgG antibody to S. sonnei O antigen was an indicator of protection against S. sonnei (Table 4). Prechallenge local IgA antibody also tended to indicate protection, but the difference in attack rates in antibody-positive vs antibody-negative persons was not significant.

DISCUSSION

Immunity to shigellosis is believed to be largely specific to the Shigella serotype, which is determined by cell surface lipopolysaccharide O antigens (1). Oral immunization with attenuated Shigella strains has provided serotype-specific protection; however, these vaccine strains have not been suitable because they are occasionally genetically unstable (4) and require annual booster doses to maintain protection (6).

It has recently been recognized that protection against enteric pathogens, like Shigella, may be determined by the production of secretory immunoglobulins at the level of the intestinal mucosa, where they may prevent the adherence of the bacterium or interfere with bacterial toxins. This recognition, along with the hypothesized importance of O antigens in stimulating a protective immune response, led to the development of a vaccine strain in which Shigella O antigen is delivered to the intestine by an attenuated enteric organism (8). This should

result in stimulation of the immune system in the gut, which has in fact been demonstrated in animal models (13).

The recipient strain S. typhi Ty21a is itself an effective oral vaccine against typhoid fever. In a field trial in Egypt, it was shown to be safe and to offer 95% protection (9), while in Chile it had approximately 50% efficacy for at least 3 years (10,11). S. typhi Ty21a may be particularly suitable as a recipient organism, because of its unique properties as an attenuated S. typhi. In the small intestine, it crosses the mucosa and is ingested, but is not immediately killed, by macrophages (1,20). Thus, the organism can bring antigens into direct contact with lymphoid tissues and may stimulate a stronger protective response than carrier strains that remain in the intestinal lumen, such as non invasive E. coli expressing Shigella O antigens, (such strains did not result in protective immunity) (21). As Ty21a can serve as a carrier of antigens from a variety of other microorganisms, the study is important not only as an evaluation of this particular vaccine strain, but also as a test of the concept that Ty21a can deliver to the gut heterologous antigens, which then stimulate a protective immune response.

In this first clinical evaluation of a prototype bivalent enteric vaccine, the S. typhi-S. sonnei strain 5076-1C provided a very substantial level of protection against challenge with S. sonnei. The 53% protective efficacy against diarrhea and 71% protection against dysentery in the first three studies, are, in fact, similar to the 64% level of protection afforded by shigel-

losis itself in similar challenge studies with S. flexneri (22). It should also be noted that the challenge inocula used resulted in ~~69% attack rate~~ for illness in controls, substantially higher than that observed in endemic areas, where the usual ingested dose of Shigella is probably lower. Since it is likely that protection is greater when the inoculum of ingested organisms is small, in comparison to when the inoculum is large, it could be predicted that the protective efficacy with the vaccine may be even higher in a field trial setting, under natural conditions of challenge. Indeed, a previous oral attenuated Shigella vaccine had 56% efficacy in volunteer challenge studies (22), but gave greater than 80% protection in field trials in endemic areas (6,23,24).

The reason the vaccine lot prepared for field trial failed to protect volunteers is unclear. The vaccine was ostensibly prepared and administered in the same way as with the two previous lots that stimulated excellent protection. Changes in the procedures necessary for production in larger scale required to provide sufficient doses for field trials may inadvertently have resulted in differences between lots. It was previously noted with another attenuated Salmonella vaccine that lyophilization had a deleterious effect (25). Ultimate application of an attenuated vaccine requires that large scale production and lyophilization be successfully accomplished, so that sufficient quantities of vaccine are available and can be stored and transported before use.

Although there are more than 30 serotypes of Shigella, four

to five, including S. sonnei, usually predominate. S. typhi Ty21a has been genetically modified to express the major somatic antigen of S. flexneri serotype 2a (26), as well as the form I antigen of S. sonnei. Furthermore, Ty21a has been engineered to express a colonization factor antigen (27) and the B subunit of enterotoxigenic Escherichia coli (28). This raises the real possibility that Ty21a can serve as a genetic carrier for a number of antigens, resulting in a combination oral vaccine, protective against typhoid fever and important types of diarrhea.

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TABLE 1

**Efficiency of *Salmonella typhi*/*Shigella sonnei*
Divalent Vaccine in Protection Against Challenge
With Pathogenic *Shigella sonnei***

Group	No. Volunteers	Any Illness	Diarrhea	Dysentery	Laxac.		Severity of Diarrhea*	
					>100 g	>101 g	Mean No. Stools	Stool Volume(ml) Positive Coproculture
Controls	36	25	20	19	20	16	9.2	1320
Vaccinees	40	17	13	9	9	4	0.2	1094
P value			0.06 ^b	0.01 ^c	0.006 ^b	0.001 ^c		

- * Portals only to those with diarrhea or dysentery
^b Fisher's exact test, one tail, controls vs. vaccinees.

TABLE 2

Comparison of efficiency in studies 1-3 (lots 2 and 5)
and study 4 (lot 8) with Salmonella typhi/Salmonella paratyphi vaccine

	No. Volunteers	Any Illness	Diarrhea	Dysentery	>100 F	Fever >101 F
<u>Study 1-3</u>						
Vaccinees	28	10*	8*	5*	4*	0*
Controls	29	20	10	14	17	13
<u>Study 4</u>						
Vaccinees	12	6	3	4	5	4
Controls	9	3	2	5	3	3

* All p<0.025, Fisher's exact test, one tail, controls vs. vaccinees

Table 3

Serum and Intestinal Antibody Rises to *Shigella sonnei* O Antigen
in *Salmonella typhi*/*S. sonnei* Vaccinees and in Vaccinees and Controls
After Challenge With *S. sonnei*

Group	Antibody Rise After Vaccination			Antibody Rise After Challenge		
	Serum IgG	Serum IgA	Local IgA	Serum IgG	Serum IgA	Local IgA
Vaccinees	12/56 (21)	14/56 (25)	6/17 (35)	9/40 (22)*	17/40 (42)*	1/25 (4)*
Controls				17/38 (45)*	26/38 (68)*	7/27 (26)*

*: $p = 0.07$, $\pm p = 0.04$, #: $p = 0.07$, chi square

Table 4

Relationship Between Serum or Intestinal Antibody Status
and Attack Rate After Challenge With *Shigella sonnei*

<u>Antibody Status</u>	<u>Attack Rates After Challenge</u>	
Serum IgG Rise After Vaccination (Yes vs No in vaccinees)	4/10 (40%)	vs 12/30 (40%)
Serum IgA Rise After Vaccination (Yes vs No in vaccinees)	1/8 (12%)	vs 15/32 (47%)
Prechallenge Serum IgG Titer (≥ 40 vs <40 in vaccinees and controls)	9/26 (35%)	vs 27/43 (63%)*
Prechallenge Serum IgA Titer (≥ 40 vs <40 in vaccinees and controls)	13/41 (32%)	vs 28/37 (76%)*
Prechallenge Intestinal IgA Titer (≥ 4 vs <4 in vaccinees and controls)	11/23 (48%)	vs 19/29 (66%)

*: $p = 0.04$, $t < 0.001$, chi square

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